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Debra J. Trantolo 5/12/97
PI - Signature Date

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Delivery of Vaccines by Biodegradable Polymeric Microcapsules with Bioadhesion Properties

Cambridge Scientific, Inc.
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Executive Summary

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The objective of this Phase II SBIR proposal is to develop a PLGA-based delivery system for oral vaccines: one of military interest and one of private sector interest. The first year of this project has focused in two areas: (1) development of the necessary technology to obtain the correct particle size of the polymer matrices; and (2) *in vitro* bioadhesion studies. A combination of impeller milling, air milling, and microfluidizer technology has returned particles of less than 10 microns which will help accelerate the uptake of antigens. The use of 10% gelatin in the formulations has increased bioadhesion considerably.

1 INTRODUCTION

1.1 Background and Rationale

Traditionally, vaccine research has been mainly concerned with the induction of systemic immunity through parenteral immunization, usually involving intramuscular or subcutaneous routes of administration. While this approach may be appropriate against diseases caused by agents which infect through punctured or damaged skin, e.g., as with malaria, it is widely known that the majority of pathogens infect through mucosal routes, such as oral, nasal, or genital (O'Hagan, 1994). Parenteral vaccines do not induce mucosal immunity, which is primarily mediated through the production of secretory immunoglobulin A (sIgA).

Despite recent advances in the understanding of the initiation, control, and maintenance of the secretory immune response, success with oral immunization has not been realized. Degradation of antigens in the gastrointestinal (GI) tract, limited absorption, interaction with nonspecific host factors, inadequate delivery systems, and pre-existing immunity have all negatively influenced the outcome of oral immunization. However, recent research has highlighted strategies which may be useful in the development of novel delivery systems for oral administration. These novel systems include live vectors, such as adenovirus, salmonella, and poliovirus, and nonreplicating antigen carriers, such as liposomes, lectins, and microparticles (the subject of this work). There are two general approaches for the development of oral vaccines: (1) genetic modification of live viral and bacterial vectors for expression of antigens from non-target microorganisms (or genetic modification of the microorganism itself rendering it non-pathogenic), and (2) formulation of antigens into nonliving carrier systems which protect against antigen degradation and target delivery to the gut-associated lymphoid tissue (GALT) itself. It is this latter area of delivery system formulation to which this Phase II SBIR project has been directed.

Accumulated experimental evidence from animal models establishes the presence of a common mucosal immune system which may be stimulated by oral immunization. Oral immunization has been shown to result in the induction of secretary IgA and T cell responses at mucosal sites. Thus, oral immunization may be used for the induction of protective immunity against pathogens of the GI tract, as well as pathogens that infect at alternative mucosal sites. However, the induction of mucosal immunity following oral immunization has been shown to depend on a number of variables, including the dose and the nature of the antigen, and the frequency of the administration. One of the most crucial factors, then, in the success of oral immunization is the selection of the antigen delivery system. The project reported on herein addresses the optimization of a microparticle delivery system for oral immunization.

The objective of this Phase II project is to expand on the Phase I results and develop a PLGA-based delivery system for induction of mucosal immunity following oral administration. The subject system has as its basis our patented matrix formulation technology which, based on its previous performance in applications to systemic delivery, may accommodate the needs of an oral delivery system. The technology relies on matrix formulation methods, rather than encapsulation methods, to produce a biocompatible,

degradable micron-sized particulate with the encompassing features of adjuvancy and bioadhesion. Encapsulated PLGA-based vaccines have been investigated for their adjuvant properties (e.g., Alonso et al., 1993; Eldridge et al., 1991a; O'Hagan et al., 1991), their size requirements (e.g., Eldridge et al., 1991b; O'Hagan et al., 1993), and their controlled release characteristics (e.g., Gilley et al., 1992; O'Hagan et al., 1993), all of which highlight the potential of a PLGA-based system. The proposed delivery system, however, differs on its basis as a *matrix* formulation which should maintain the advantages of the cited systems while offering other advantages. Using integrated data derived from the Phase I SBIR project as well as other applied projects, it is the expectation that the proposed system will more likely preserve protein antigenicity during formulation (biologicals are dispersed within the polymer matrix using aqueous or other stabilizing media) and more easily adapt to incorporation of bioadhesives (in systems where enhanced adhesion may augment the immune response). In addition, our methodology (1) yields dose forms with high batch-to-batch reproducibility, a factor which favors regulatory approvals, and (2) has been patented by Cambridge Scientific, Inc., a factor which should contribute to the commercial potential of an SBIR project undertaken.

1.2 Project Objectives

During Phase I of this SBIR, an improved method for the delivery of antigens to the mucosal associated lymphoid tissue (MALT) was developed. Uptake of antigen is a critical initial step in the generation of mucosal immunity, based on the stimulation of IgA antibody secreting cells and helper T cell subsets in the lymphoid follicles of the gut and other mucosal tissues [Owen and Ermak, 1990; Neutra and Kraehenbuhl, 1992]. IgA antibodies secreted at mucosal surfaces represent the principal barrier to colonization and invasion of the host by a wide variety of microorganisms and viruses, and a mechanism for neutralization of toxins produced by microorganisms or administered by artificial means. For efficient induction of mucosal immunity it is necessary to present antigens in particulate form to specialized microfold (M) cells, which are present at highest density in follicular domes of the MALT, and are known to bind the transport particles in the size range of 10 nm to 10μm in size [Owen and Ermak, 1990; Neutra and Kraehenbuhl, 1992; Lefevre et al., 1985; and Pappo and Ermak, 1989]. Many mucosal antigens, by themselves, are not of a size which is optimal for uptake by M cells. For example, *H. pylori* urease, (an antigen used extensively by our collaborators at OraVax, Inc.) one of the subject antigens, is a multimeric structure composed of 6 copies of the individual structural proteins and has a particle size of about 12 nm [Goodwin et al., 1989]. In addition, it is uncertain whether these types of biological antigens will be degraded by intestinal enzymes, or whether protection of the antigen by a polymer until it is delivered to Peyer's patch lymphocytes may enhance antigen preservation and presentation to the immune system. Thus, for induction of IgA immunity, we have set out to "package" antigens in particulate formulations that optimize their association with M cells. Once bound and internalized by M cells, a variety of other parameters of the antigen formulation may determine the anatomical fate of the antigen, its processing by antigen presenting cells, and the rate at which antigen is released. In general, preservation of the

particulate nature of antigens after uptake/internalization and the slow release of antigen from biodegradable particles have been associated with significant adjuvant effects [Eldridge et al., 1991].

Despite the fact that the MALT is the predominant immunological system of the body, the process whereby particulate antigens are bound and transported by M cells is relatively inefficient, and it is estimated that only 0.01-1.0% of particles presented to the MALT are taken up [Owen and Ermak, 1990; Neutra and Kraehenbuhl, 1992; and Lefevre et al., 1985]. Various properties of particles affect binding and uptake by M cells, including charge and hydrophobicity. The rate and process whereby particulates transit the mucosal surface may also affect M cell binding and uptake. An important objective of the Phase I project was to develop particulate formulations with bioadherent surface characteristics that will increase the efficiency of uptake by M cells and result in enhanced IgA responses at mucosal surfaces.

2. PHASE II TECHNICAL OBJECTIVES AND APPROACH

Traditionally, vaccine research has been mainly concerned with the induction of systemic immunity through parenteral immunization, usually involving intramuscular or subcutaneous routes of administration. While this approach may be appropriate against diseases caused by agents which infect through punctured or damaged skin, e.g., as with malaria, it is widely known that the majority of pathogens infect through mucosal routes, such as oral, nasal, or genital (O'Hagan, 1994). Parenteral vaccines do not induce mucosal immunity, which is primarily mediated through the production of secretory immunoglobulin A (sIgA).

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The objective of this project is to develop a PLGA-based delivery system for induction of mucosal immunity following oral administration. The subject system has as its basis our patented matrix formulation technology which, based on its previous performance in applications to systemic delivery, may accommodate the needs of an oral delivery system. The technology relies on matrix formulation methods, rather than encapsulation methods, to produce a biocompatible, degradable micron-sized particulate with the encompassing features of adjuvancy and bioadhesion. Encapsulated PLGA-based vaccines have been investigated for their adjuvant properties (e.g., Alonso et al., 1993; Eldridge et al., 1991a; O'Hagan et al., 1991), their size requirements (e.g., Eldridge et al., 1991b; O'Hagan et al., 1993), and their controlled release characteristics (e.g., Gilley et al., 1992; O'Hagan et al., 1993), all of which highlight the potential of a PLGA-based system. Our delivery system, however, differs on its basis as a *matrix* formulation which should maintain the advantages of the cited systems, while offering other advantages. Using integrated data derived from Phase I of this project as well as other applied projects, it is the expectation that the proposed system will more likely preserve protein antigenicity during formulation (biologicals are dispersed within the polymer matrix using aqueous or other stabilizing media) and more easily adapt to incorporation of bioadhesives (in systems where enhanced adhesion may augment the immune response).

The project focuses on developing two oral vaccines- one of commercial interest and one of military interest. One potential commercial candidate is an oral vaccine designed to deliver *Helicobacter pylori* urease. *Helicobacter pylori*, a gram-negative bacterium is one of the most prevalent infections worldwide, causing infection of over 50% of adult populations in developed countries and nearly 100% in developing countries. There is now general agreement that *H. pylori* is the cause of chronic superficial (type B) gastritis and is strongly associated with the majority of cases of peptic ulceration. An increasing body of evidence also exists for an etiologic role of *H. pylori* infection in atrophy of the gastric mucosa, adenocarcinoma, and non-Hodgkin's lymphoma of the stomach. For these reasons, there has been considerable interest in the development of vaccines as a potentially cost-effective approach to the prevention of Helicobacter-induced chronic diseases. OraVax, Inc., our collaborator in the Phase I aspects of this SBIR, as well as in other pilot studies, has as one of its development objectives the development of an oral vaccine for prevention and treatment of *H. pylori* infection. The collaboration has continued in Phase II, and *H. pylori* urease continues to be a commercial candidate for oral vaccine development. This first choice system addresses private sector SBIR commercial development objectives.

The second oral vaccine will be based on a candidate vaccine antigen as supplied by the USAMRIID. In initial discussions, Venezuelan Equine Encephalitis (VEE) is projected to be this candidate (and will be used in the text of this report in reference to the

Army-selected candidate). Final selection will be made at contract negotiation following consultation with Army technical staff. This second candidate system will address commercial development objectives relating to Government use.

Each of the candidate antigens will be incorporated into a PLGA-based biodegradable carrier. Based on Phase I results, two types of PLGA matrices will be prepared, one combining antigen with PLGA exclusively, the other combining antigen with PLGA and gelatin. The former will exploit those considerations of particulate size and antigen controlled release; the latter will address the additional potential of matrix bioadhesion. Initial formulations continue with the 75:25 PLGA copolymer used in the feasibility studies with antigen loadings in the range of 1%, also as used in Phase I. Potencially, if immunogenicity studies indicate a need to alter the antigen release characteristics, other copolymer ratios (higher glycolide ratios increase polymer degradation thereby increasing antigen release) and other antigen loadings (higher antigen loadings generally increase the rate of antigen release) will be considered.

Having demonstrated in Phase I that antigenicity is retained in the particulate formulations and that gelatin enhances bioadhesion and particle uptake in a model poly-lysine/PLGA matrix, the first year of the Phase II workplan has focused on confirmatory development by establishing the actual optimization of *in vivo* particle uptake by the bioadhesive-added and bioadhesive-free PLGA/antigen. These studies will utilize the ligated intestinal loop protocols developed at OraVax, Inc. for the Phase I uptake studies (see also Ermak et al., 1995, for reference). These studies will establish the desired particle sizing characteristics of the PLGA-only system vs. the PLGA/bioadhesive system, thus focusing on the relative merits of each in further evaluations.

The second year of the proposed development plan will then establish the dose/response characteristics for the development of an immune response. Matrices corresponding the commercial interest will be evaluated at Cambridge Scientific, Inc. using mice as the *in vivo* tool (corresponding to the test system in place at OraVax, Inc. for urease testing). Analyses of the immune response will be based upon serum and secretory antibody responses to antigen by enzyme immunoassay (ELISA). VEE matrices will be evaluated at the USAMRIID using Army-developed protocols.

The final facet of the Phase II development will be the optimization of protective immunity in response to administration of the oral vaccine using challenge testing conditions. Commercial matrices again will be evaluated at Cambridge Scientific, Inc. using mice protocols developed by OraVax, Inc. Fourteen days after completion of the immunization phase, mice are challenged by intragastric administration of virulent organisms. Two weeks later, mice are euthanized and autopsied; testing for antigen follows spectrophotometric methods, as well as tissue visualization protocols. VEE matrices will be evaluated at USAMRIID, again using Army-developed procedures most likely involving aerosol challenge tests.

It is anticipated that the outcome of the Phase II project will be the definition of a viable PLGA oral vaccine delivery system for both a private sector and a government interest. Phase III plans will address the regulatory development of the defined systems within the context of a final FDA-approved dose form.

3. PHASE II WORK PLAN

3.1 Introduction

Microspheres and microcapsules made of poly (D,L-lactic-co-glycolic acid) (PLGA) have been investigated as vaccine delivery systems [Eldridge et al., 1991; Eldridge et al., 1991; and Singh et al., 1991]. Vaccine-loaded PLGA microparticles have provided continuous antibody titer against infectious agents for up to 120 days, eliminating the need for repeated administration [Eldridge et al., 1991]. In addition, vaccine-loaded microparticles can be administered either as an injectable or as an oral formulation for systemic or mucosal immunity.

Most formulations of vaccine-loaded PLGA microspheres and microcapsules are prepared using organic solvents such as methylene chloride [Eldridge et al., 1991; Singh et al., 1991]. When Cambridge Scientific, Inc. was founded, we focused on developing a delivery system which does not require organic solvents for incorporation of biologicals. While initially provoked by client concerns, there are several reasons favoring our progress in establishing this proprietary area. First, methylene chloride, an organic solvent commonly used in microencapsulation protocols, is a known carcinogen. Additionally, residual solvents have plasticizing effects which effect the reproducibility of release. Moreover, organic solvents used for microsphere preparation can denature the antigen and render it biologically inactive. PLGA microcapsules prepared by solvent evaporation methods are not very effective for controlled delivery of vaccine [Singh et al., 1991]. The encapsulated vaccine is released rapidly upon hydrolysis of the wall polymer. Our patented procedure may obviate some of these concerns while accommodating the attractive features of PLGA microcapsules. Since the delivery system uses aqueous solutions for incorporation of biologicals, it is expected to release the antigen continuously, have no stability problems, and be inherently safe for human use.

The design of an oral vaccine system should enable an increase of the fraction of antigen absorbed by the Peyer's patch, estimated to be in the neighborhood of 1%. Ideally the properties of such a system should be as follows:

1. It should pass through the stomach and to the ileum without significant loss of active agent.
2. It should develop bioadhesive properties at the pH of the ileum, and
3. It should have a particle size of < 10μm.

In the first year of the Phase II project, microparticles were prepared at 1% (w/w) loading of antigen. One set of particles will contained PLGA and antigen only; another set of particles will additionally contained gelatin. Initial studies weighed controlled release behaviors of the preparations against the bioadhesive characteristics, and thus varied gelatin content in the first round of optimization. Based on Phase I results, gelatin loadings covered the 1-10% range with *in vitro* release and bioadhesion studies used to identify likely *in vivo* formulation candidates.

3.2 PHASE II TASK OUTLINE

Task 1: Selection and Preparation of Poly(Lactide-co-Glycolide) Excipient

The poly(lactide-co-glycolide), PLGA, initially proposed for Phase II is based on Phase I results. Degradation rates depend on the ratio of monomers, being most rapid for a PLGA's having mole ratios of lactide to glycolide between 1:3 and 1:1 [Cutright et al., 1974]. This aspect of formulation can later be modified based on test results.

The polymer for this study is a 75:25 PLGA. This is commercially available (e.g., from Boehringer Ingelheim through Henley Chemicals, Inc.). Upon receipt, the polymer is characterized with respect to molecular weight and molecular weight distribution using gel permeation chromatography (GPC) using a Waters Associates GPC equipped with a 600E System Controller, a 410 Differential Refractometer, a 710 WISP autosampler, and an 825 Maxima software package for data handling. Separations are accomplished on two columns placed in series, an "Ultrastyragel 100 Angstrom" (Waters) for high MW polymer and an "Ultrastyragel Linear" (Waters) for low MW polymer.

A low density polymer foam is then prepared by lyophilization of a polymer solution. The polymer foam is cryogenically ground in a Tekmar Model A-10 Analytical Mill equipped with a cryogenic well enabling particle size reduction at liquid nitrogen temperatures (-196°C). Grinding for 8-10 minutes at 20,000 rpm following by sieving in a mechanical shaker equipped with Taylor sieves allows the porous foam polymer to be distributed among discrete particle size ranges (e.g., < 45 μm, 45-90μ,m, 90-125μm, 125-180μm, and >180μm). In our formulation process, the particle size of the polymer with which the active agent is blended (as opposed to the particle size to which the extruded matrix is later reduced) has a controlling effect on release rates. Starting polymer foam particle size ranges are 125-180μm for this study. Final matrix particle sizes is < 10μm. Particle size distribution will be confirmed with a Micromaster Model CK microscope equipped with a size calibration grid.

Task 2: Preparation of PLGA Matrices

To insure homogeneous blending of the PLGA and the protein antigen, an aqueous solution of the antigen is absorbed into the polymer in a concentration to permit a final antigen loading in the PLGA matrix of 1%. Bioadhesive matrices contain 1, 5, and 10% gelatin. To prevent migration of the protein to the surface, subsequent removal of the water is accomplished by freeze drying. The matrix is compressed and extruded at a pressure of about 15-20,000 psi which permits the process to occur at temperatures of about 45-55°C. High pressure extrusions insure that the protein is fully incorporated within the polymer lattice with a concomitant reduction in particle porosity; this minimizes premature release of the active agent. The sized PLGA/protein blend is loaded into a mold and extruded under pressure through a 2.4 mm diameter die using a Compac Type MPC 40-1 hydraulic press (Stenhoj Co., Denmark). This press allows extrusions to be carried out at constant pressures and temperatures. Temperature is monitored by thermocouples attached to an Omega Type DP 460 Digital Thermometer. Following extrusion the matrix is again cryogenically ground first in a Tekmar hammer mill, then in a Trost air mill to particle sizes of less than 10μm. To determine whether the formulation

process perturbs the physicochemical and antigenic properties of the antigen, antigen recovered from particles after hydrolysis in PBS will be characterized by ELISA.

Task 3: Determination of In vitro Bioadhesion

The experimental design is an adaptation of the method described by Chickering and Mathiowitz (1995). These workers used an electrobalance to investigate bioadhesion of spherical polymeric microspheres to rat intestinal mucosa. We have used small pressed cylindrical tablets of our PLGA/bioadhesive formulations (2.0 mm diameter). These are suspended from one surface by a fine wire into a temperature controlled cell containing a section of rat intestine cut longitudinally to expose the lumen. The section is attached to the bottom of the cell and bathed in phosphate buffered saline (PBS). The wire, in turn, is suspended from the weighing arm of a Roller-Smith Precision Balance (Rosano Surface Tensiometer, Biolar Corp., North Grafton, MA). This configuration allows the opposite end of the cylinder to be pressed into the mucosa with a force which can be varied up to the weight of the tablet less the buoyant force exerted by the medium on the tablet. After contact between the tablet face and mucosa for a predetermined time (1 minute), the tablet is slowly raised and the force required to break the contact is registered on the tensiometer scale.

The adhesive force is determined by the area of contact as defined by the cross-sectional area of the tablet. Thus, the adhesive force per unit area is given by

$$F = (\Delta w)\, g \,/\, \pi R^2 \qquad \text{dyne / cm}$$

where

Δw = difference between the tensiometer reading at rupture of the adhesive bond and the contact force, grams
g = gravitational constant, cm/sec^2
R = tablet radius, cm

The goal of this task was to determine the minimum gelatin requirements for statistically significant enhancement of bioadhesive characteristics of the formulations.

Task 4: Determination of In vivo Uptake of Particles

Particle uptake experiments were conducted using ligated mouse intestinal loops. Mice (Swiss-Webster, Female, 7-9 weeks) were fasted overnight prior to surgery, with H_2O provided ad libitum. Sodium pentobarbital (50 mg/kg) will be administered IP using a 27 gauge needle to anesthetize animals. Abdomens will be swabbed with 70% ethanol and the abdominal cavity will be opened by a ventral midline incision. A Peyer's patch free of obvious fecal matter will be located, and one end ligated, allowing blood circulation to remain intact. Another ligature was prepared opposite the Peyer's path, but it will not be tied off. Microparticle formulations (10^8-10^9/loop) in 100μl of PBS (pH 7.2) were instilled in each loop through the loose ligature of the loop, immediately followed by tightening of the ligature. The total intestinal loop length is 3-4 cm. The loop was placed back into the abdominal cavity and the incision sutured. After 1-2 hours,

the animals will be euthanized and the ligated loops removed, washed and processed for electron microscopy by fixation in a solution of 2.5% glutaraldehyde, 2% formaldehyde, and 0.1M cacodylate buffer (pH 7.4). Tissues were post-fixed in 1% OsO_4 in 0.1M cacodylate buffer (pH 7.4), stained en block in uranyl acetate, and embedded in Epon/Araldite. Thin sections were examined using an AMR-100 scanning electron microscope (Amray, Inc.)

Task 5: Evaluation of Immunogenicity of Urease Matrices

The efficiency of oral immunization with different particulate formulations will be assessed in outbred (Swiss) SPF mice. The identified formulations, standardized with respect to antigen content, will be delivered by intragastric gavage to groups of five 4-5 week old mice over an antigen dose range of 5, 25, and 125 μg. (In previous dose response studies employing free urease, OraVax has demonstrated significant protection at doses of 30 μg). Antigens will be administered on days 0, 7, 14, and 28. Each formulation will be co-administered with *E. coli* heat-labile toxin adjuvant (LT, 5μg/mouse) or without LT. Control groups will receive (1) a dose (125μg) of soluble urease with LT; and (2) placebo PLGA particles with LT. Mice will be tested for serum and secretory antibody responses to urease by enzyme immunoassay (ELISA). Sera, saliva and feces will be collected prior to immunization and 7 days after the last immunization; samples from individual mice will be tested. Salivary samples are obtained from mice after subcutaneous administration of pilocarpine (5 mg/kg) and sorted at -20°C. Fecal pellets are frozen at -20°C until tested, and IgA extracted by addition of phosphate buffered saline (pH 7.2) with 5% nonfat dry milk and a cocktail of protease inhibitors (AEBSF, leupeptin, aprotinin and betatin). All samples from an individual animal are tested in batch mode to avoid interest variation.

Feces and saliva will be tested at starting dilutions of 1:4, and plasma at 1:100. Samples are diluted in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 (PBS-BT). Tests for antibodies of the IgG and IgA classes in sera, feces and saliva will employ a direct ELISA, in which antigen is bound to microtiter plates, blocked with PBS containing 1% BSA, incubated with duplicate 2-fold dilutions of antibody sample followed by biotinylated goat antibody against the respective immunoglobulin, and then by streptavidin-peroxidase and ABTS substrate. Specific antibody concentrations in samples are determined by interpolation from standard curves using control sera with known antibody concentrations. Since immunoglobulin concentrations in feces and saliva samples are variable, specific antibody activity in these samples will be adjusted with reference to total immunoglobulin content and expressed as specific Ig/μ g total Ig. Differences in dose response between formulation groups will be analyzed by multiple regression.

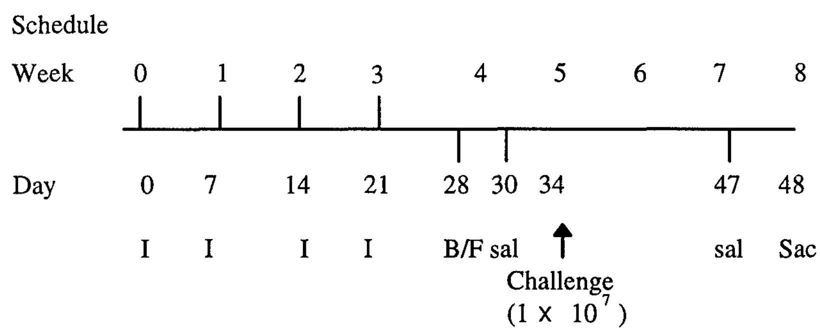
Task 6: Evaluation of Immunogenicity of VEE Matrices

Samples of the VEE/PLGA and VEE/gelatin/PLGA matrices will be submitted to USAMRIID for evaluation of immunogenicity using Army-developed protocols. It is anticipated that these studies will also be done using mice as the *in vivo* model.

Task 7: Evaluation of Protective Efficacy of Urease Matrices (Oral Challenge)

Fourteen days after completion of the immunization phase, mice will be challenged by intragastric administration of 10^7 *pylori* organisms. Two weeks later, mice are euthanized and autopsied. A longitudinal section of 1/3 of the stomach is tested for antigen by spectrophotometric assay [Hamilton-Miller and Gargan, 1979]. One-third of the stomach is fixed in 10% formalin, embedded in paraplast and sections examined after staining with Warthin-Starry silver stain and hematoxylin-eosin. The remaining third of the stomach is frozen for other studies (e.g. PCR). [Culturing for *H. pylori* is not a useful method in non-germfree mice.] The proportion of mice infected (as defined by Warthin-Starry stain and antigen) is determined in each group, and differences compared by Fisher's exact test.

The experimental plan for immunization and challenge is presented in Figure 4.



I Immunize S/web mice with rURease (lot 94J03)/PLGA reconstituted in RO/DI water, by the oral route with or without 5ug LT.

B Blood to be collected for anti-urease antibodies.

sal Saliva collected for anti-urease IgA

C Challenge with 1×10^7 H. pylori intragastrically. Animals should be fasted throughout the day and given $100\mu l$ 2% $NaHCO_3$ prior to challenge in the afternoon.

Sac Sacrifice 2 weeks (day 48) after challenge. Blood and feces collected. All stomachs harvested for formalin fixation for histology (1/4), urease activity in intact tissue (1/8, antrum; 1/8, corpus), homogenized in 1.5 ml brucella broth for quantitative culture/capture ELISA/PCR (1/4) and frozen for immunohistochemical staining (1/4).

Figure 1 Immunization with *H. pylori* rUrease/PLGA as a dose response and protection from an *H. pylori* challenge in the mouse model.

Task 8: Evaluation of Protective Efficacy of VEE Matrices (Aerosol Challenge)

Samples of the VEE/PLGA and VEE/gelatin/PLGA matrices will be submitted to USAMRIID for evaluation of protective efficacy upon aerosol challenge. It is anticipated that these Army protocols will continue to use mice as the *in vivo* model.

4. PHASE II MATERIALS AND METHODS

4.1 Reagents

- *Protein Model (for uptake studies)*

  Poly(L-lysine) FITC labeled. (Sigma Chem. Co. Cat. No. P3069, lot 63H5003). The fluorescent label, FITC, fluorescein isothiocyanate, is present in a ratio of 0.004 moles FITC per mole L-lysine monomer. The reported degree of polymerization if 219 (LALLS), the molecular weight is 45,700 (LALLS).

- *Bioadhesive*

  Gelatin, type A, from porcine skin, approximately 300 bloom (Sigma Chem. Co., Cat. No. G2500, lot 54H0724

- *Polymers*

  Poly(lactide-co-glycolide)-75:25 (PLGA-75:25), Resomer RG 756, Boehringer-Ingelheim, Article 640670, lot 241031.

  Poly(lactide-co-glycolide)-50:50 (PLGA-50:50), Resomer RG 506, Boehringer-Ingelheim, Article 640664, lot 34034.

  Poly(lactide-co-glycolide)-50:50 (PLGA-50:50), Resomer RG 502, Boehringer-Ingelheim, Article 640667, lot 241846.

  Poly(lactide-co-glycolide)-50:50 (PLGA-50:50), Resomer RG 503, Boehringer-Ingelheim, Article 640661, lot 34033.

- *Solvents and Chemical Reagents*

  All standard solvents and reagents were obtained from reliable suppliers and were used as received.

4.2 Polymer purification and characterization

PLGA-75:25 was purified as follows. Fifteen (15) grams were dissolved in 200 ml acetone and slowly precipitated by pouring into a large excess of isopropanol (IPA). Fresh IPA was used when the IPA became cloudy. The fibrous precipitate was drained, air dried, and then vacuum dried at <1 mm Hg. The yield of purified polymer is generally in the range of 70-75%. Polymer molecular weight is currently being verified by gel permeation chromatography (GPC).

The various 50:50 PLGA polymers are not soluble in common purification solvents (e.g., acetone). The polymers were obtained as GMP pharmaceutical grade and used as received.

All polymers are presently being characterized by gel permeation chromatography (GPC) for molecular weight and molecular weight distribution. The GPC system uses Waters instrumentation and is equipped with a 600E system controller, a 410 Differential Refractometer, a 710 WISP autosampler, and an 825 software package for data handling. Separations utilize two columns placed in series, an "Ultrastyragel 100 Angstrom" (Waters) for high molecular weight (MW) polymer and an "Ultrastyragel Linear" (Waters) for low MW polymer. Analyses typically use THF as the mobile phase. The 50:50 PLGA polymers, however, are only sparingly soluble in THF. We presently have a new column on order to allow analyses of the 50:50 polymers in another solvent, e.g., methylene chloride.

GPC analyses of polymers uses polystyrene standards. The reported average molecular weight of Resomer 502 is (nominally) 10,000; that of Resomer 503 is 40,000; that of Resomer 506 is 100,000; and that of Resomer 756 is ~100,000.

4.3 Poly(L-lysine)-FITC, (PLL-FTIC), Spectral Characterization

The spectrum of PLL-FITC was taken in phosphate buffered saline (PBS) on a Varian Assoc. Cary-1 UV/vis spectrophotometer. A calibration curve was established in the concentration range 0-48.5 mg/ml. The absorbance data is presented in Table 1. The calibration curve gives an intercept of -0.00058 and a slope (extinction coefficient) of 3.4067 absorbance units per unit concentration of 1 mg/ml (i.e., 3.4067 $AU/mg \bullet ml^{-1}$). The correlation coefficient is 0.99987.

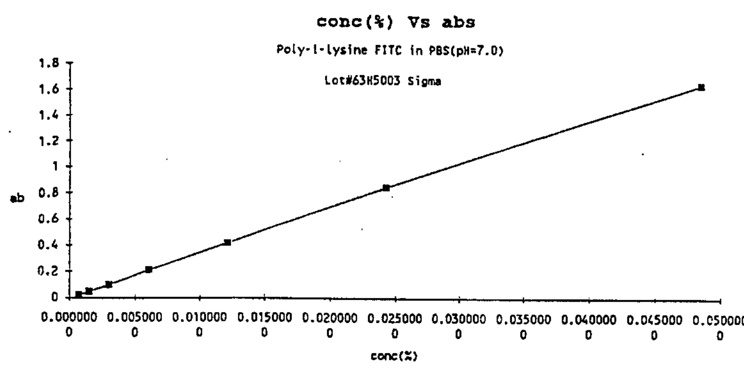


Figure 2 Poly(L-Lysine) FITC Calibration Curve

4.4 Preparation of Polymer Foams

A solution of the purified PLGA-75:25 (7.5 grams/150 ml = 50 mg/ml) was frozen and lyophilized to form an open-celled capillary-structured foam. The density of this foam was determined by measuring the dimensions of foam parallelopipeds cut from the original block.

The lower molecular weight 50:50-PLGA's can be more brittle and thus initially appeared to be viable candidates for matrix preparation because their brittleness would enhance micronization. These lower MW polymers, though, do not as easily support the foam morphology so important to the formulation protocols. The higher molecular polymer, Resomer 506, easily dissolves in glacial HAc, and maintains a rigid foam upon lyophilization. Foams formed from Resomers 502 and 503, both of which dissolve easily in HAc, collapse or flake. While there is ongoing work in this area, results-to-date suggest that we continue with Resomer 756, the 75:25 PLGA, to take advantage of Phase I results.

We are now continuing with the formulation of protein-loaded samples for *in vitro* release and adhesion studies using Resomer 756.

The foam is loaded with the active agent. In the release and adhesion studies, the foam will be loaded simultaneously with the FITC-PLL and with the type A gelatin. The foam is immersed in a co-solution containing both materials at appropriate concentrations and forced into the pores of the foam by a series of brief degassings and repressurizations. The excess solution is decanted and the solvent (water) removed by a second lyophilization. The solids content is calculated from the weight of foam before and after the second lyophilization. The appropriate solution concentrations are determined by the following equation:

$$f = [1 + d_p d_f/\{C(d_p - d_f)\}]-1$$

where f = weight fraction (loading)
d_p = absolute density of polymer, mg/cm^3
d_f = density of polymer foam, mg/cm^3
C = concentration of agent in solution, mg/cm^3

4.5 Particle Size Reduction

Efficient absorption of the particulate matter which comprise the oral vaccine formulation depends on particle size distribution. The Peyer's patches of the ileum are capable of engulfing by pinocytosis particles of 10 μm or less.

The final steps in production of the oral dose form involve grinding the compact extruded matrix. Particle size reduction of PLGA composites to micron level sizes represents a significant unit operation in the manufacture of a matrix-based oral vaccine. We have arbitrarily established an immediate goal of 100% of the particles to be <45 μm (325 mesh) or less. If the distribution is calculated assuming all particles to be spherical and either 10 or 45 μm in diameter, the yields calculated on a weight basis are as follows:

Number Distribution	Weight Percent at 10 μm
90% at 10 μm; 10% at 45 μm	8.99
95% at 10 μm; 5% at 45 μm	17.25
99% at 10 μm; 1% at 45 μm	52.07
99.9% at 10 μm; 0.1% at 45 μm	91.64

Assuming distributions such that the mean particle size in the 10 μm in the <10 μm range is 5 μm, and that the mean diameter of the 10-45 μm range is 27.5 μm, the calculated yields on a weigh basis are even poorer:

Number Distribution	Weight Percent at 10 μm
90% at 5 μm; 10% at 27.5 μm	5.13
95% at 5 μm; 5% at 27.5 μm	10.25
99% at 5 μm; 1% at 27.5 μm	37.31
99.9% at 5 μm; 0.1% at 27.5 μm	85.72

This demonstration defines the problem of particle size reduction, as well as our immediate goal; we anticipate improving microparticulate yields by adjusting values of the milling parameters.

The particle size distribution is determined by two sequential measurements. First, the ground material is sieved through a set of standard Tyler mesh screens (80, 120, 170, and 325 mesh corresponding to 180, 125, 90, and 45 um). Sieving is standardized by shaking for 30 minutes on an electric vibrator (Syntron Electric Vibrator, Type TSS-15, Syntron, Inc., Homer City, PA). Weights of the corresponding fractions are recorded. The fines (<45 um) are examined microscopically with calibrated optics to determine the number fraction of particles <10 μm. Scanning electron microscopy is also utilized in determining the dimensions of the particles.

Our approach to particle size reduction is a three-step process. These steps are described below.

- *Impeller Milling*

First, the compact extruded matrix is cryogenically ground in a Tekmar A-10 analytical mill (Glen Mills, Inc., Clifton, NJ). This mill, cooled with liquid nitrogen, operates at 20,000 rpm and produces a grind of, according to product literature, of about 200 μm. We have routinely used this mill to produce PLGA fractions of 45-90, 90-125, and 125-180 μm. The major portion of the grind is, of course, >90 um. The fines, <45 μm, usually are a small fraction of the total. Repeated grinding with circulating coolant will maximize the 90-180 μm yield, a feed suitable for the second step.

- *Air Milling*

The yield from the first grinding step is the feed supplied to a fluid energy Trost jet mill (Model Gem-T, Garlock Plastomer Products, Inc., Newtown, PA). A schematic diagram of this mill is shown in Figure 1. Product literature indicates an appropriate feed size of 20 mesh or finer (<850 μm), well within the capability of the Tekmar A-10. Air (or jet) mill operation requires optimization of several important parameters:

1. position of the P- and O-jets;
2. pressure supplied to each jet; and
3. sample feed rate.

The position of the jets determines the vacuum at the hopper which in turn pulls the feed into the jet stream. It also affects the region of particle impact. The pressure determines the fluid (either compressed air or nitrogen) energy or velocity imparted to the particles as kinetic energy, and thus the force with which particles collide. The feed rate, if too rapid, will clog the P-tube and result in blow back (and loss) of feed. If too slow, insufficient collisions per unit time will occur, resulting in inefficient and slow grinding.

Experiments are currently being conducted with poly(vinyl alcohol), 88% hydrolyzed, and a molecular weight of 70,000 D (Polysciences, Inc.). It is useful to use a model polymer to establish operating conditions, rather than the considerably more expensive PLGA.

Veldkamp (1980) has described jetmilling theoretically in terms of plastic deformation and fracture on collisions between spherical particles. The resulting impact contact stress depends on the velocity, particle size, density, and stiffness of the material. He points out that for jetmilling, material hardness and critical stress intensity factor are essential material properties.

Cracking will start, roughly, at a threshold contact force given by

$$F = LK^4E^{0.4}/H^3$$

where L = constant
K = critical stress intensity factor
H = indentation hardness
E = stiffness

Both hardness and stiffness increase with decreasing temperature. Although an increase in the latter increases the contact force necessary for cracking, F is much less strongly dependent on stiffness, E, than on hardness, H, which appears in the denominator as a cubic term. Thus the equation predicts that contact force should decrease with decreasing temperature.

We have modified the jet mill to allow cooling. We have connected a 3-way Claisen adapter to the feed hopper. This modification has proven to have a dual value. First, it has also eliminated the problem of material loss due to blow-back when the P-tube occaisionally becomes clogged. Even if blow-back occurs, the adapter is tall enough so that expelled feed falls back into the hopper.

The second arm of the adapter is connected to a liquid nitrogen reservoir. Thus we can cool the mill prior to as well as during operation. We have monitored the temperatures at the P-jet inside the hopper as low as -60oC while passing liquid nitrogen into the mill.

- ***Microfluidization***

To insure complete reduction in particle size, Cambridge Scientific, Inc. has enlisted the help of Microfluidics International Corporation (Newton, MA). Particles with sizes less than 10 μm have been obtained through the use of Microfluidics patented technology. This system couples an intensifier pump whose long slow output stroke and quick intake stroke attain a constant pressure. The ceramic interaction chamber is devoid of moving parts and cooled with either an ice bath or cryogenically cooled. PLGA (after undergoing original size reduction in the mill and air mill) is placed in isopropyl alcohol and pressure-fed through microchannels. As the process stream is propelled forward, it separates in two, changes direction, and collides with itself into a single stream. Powerful forces of shear, impact, and cavitation occur improving the yield for the required size. A schematic representation of the process is presented in Figure 2.

4.6 *In vitro* Bioadhesion

The *in vitro* bioadhesion testing is based upon a modification of the method described by Chickering and Mathiowitz (1995). The testing is accomplished by using Roller-Smith Precision Balance (Rosano Surface Tensiometer, Biolar Corp., North Grafton, MA.). The testing is performed using a section of the small intestine (ileum) of the rat, that portion containing the Peyer's patches. Again, the Peyer's patches are the immune organ that is targeted for the release of the peptide.

The experimental technique is complex due to the nature of measuring sensitive adhesive forces. The Roller-Smith Balance is a microbalance that is able to precisely measure surface tensions. The balance consists of a moving stage and a tension dial that can measure up to 500 mgs of force. In the previous modification that was used in the Phase I work, a human hair was hung on the right side of the balance; presently, a rigid wire is being used. A glass bead is fastened to the wire using ethyl cyanoacrylate. Because of the large weight on the right-hand side of the balance, a counterweight is needed in order to zero the balance. The counterweight consists of a number of different metal wires. The counterweight is not exact, but the difference in the weights of the right and left side of the balance is made up for by turning the tension dial of the balance a certain distance.

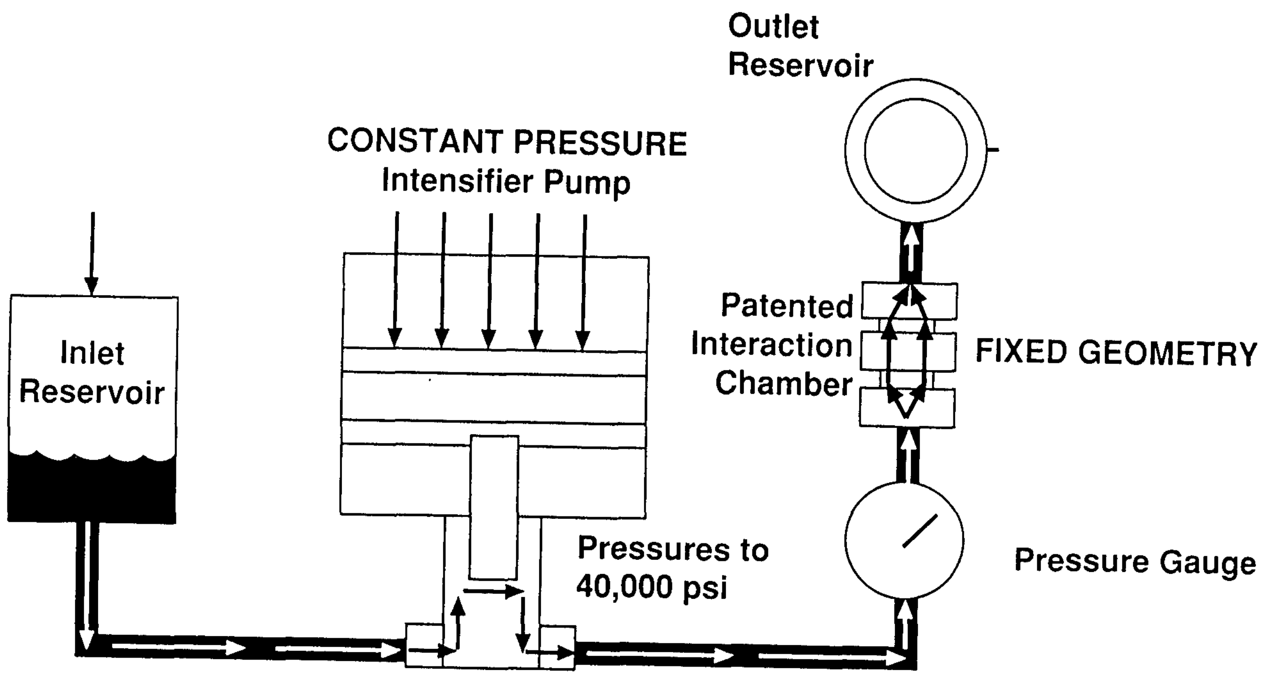


Figure 2. Schematic Representation of Microfluidics International Corporation Microfludizer (Reproduced with Permission).

The distance the dial is turned (in milligrams of force) would equal difference experienced between the right and left side of the balance. The balance is now able to be zeroed and can precisely measure the amount of adherence force.

A section of the rat ileum was excised and the outer portion of the intestine was fastened to a glass slide, thus exposing the lumen to the adherent force. The intestinal sample was rectangular and the four corners were glued to the glass slide using ethyl cyanoacrylate. The sample/slide was then immersed into a beaker containing a solution of PBS that mimics the microenvironment of an *in vivo* intestine.

After the intestine is prepared and the balance is zeroed, the bioadhesion measurements were done. The test matrix was placed onto the rigid wire via the glass bead. The polymer disc was fastened to the apparatus using ethyl cyanoacrylate. Once this was accomplished the polymer disc was lowered to about 2-3 centimeters above the lumen of the intestine that is submerged in the PBS solution. This was done by moving the mobile stage of the balance. Once the polymer disc was stabilized in the PBS solution the balance was zeroed again. The apparatus was then lowered until the polymer disc just touches the lumen of the intestine. The balance was, again, tared so that the polymer disc did not exert any pressure on the lumen. The balance was locked, and the tension dial was turned clockwise in order to provide a certain amount of force that was exerted on the right hand side of the balance. This force was in the range of 40 milligrams of force. The balance was then unlocked and the 40 milligrams of force was exerted upon the lumen. The polymer disc was allowed to adhere to the lumen for approximately five minutes.

At the predetermined time, the tension dial was turned counterclockwise in a uniform manner. This counterclockwise turning provided the adherent tension that we were seeking. The tension pulled the disc from the lumen and the force that enabled the disc to break free from the lumen was recorded.

As we exited the Phase I SBIR, we had shown that the *in vivo* uptake PLGA microparticulates could be significantly enhanced when prepared with a bioadhesive. Recall that in an oral system uptake and the induction of mucosal immunity centers optimal with PLGA particles < 10mm, even then only ~ 1% of the particulate is taken up. There are no published data relating efficiency of particle uptake with the onset of immunogenicity; however, it is commonly assumed that an increase particle uptake will increase the initial immune response and, more importantly, insure the development of protective immunity.

It is our central premise that the use of readily available bioadhesives in a PLGA matrix preparation will enhance particle uptake. Thus, while it would be desirable to ultimately have all particles sticky and < 10mm, our first goal in year one was to determine the relationships between stickiness, particle uptake, and the development of the immune response.

5. RESULTS AND DISCUSSION

5.1 *In Vitro* Bioadhesion Studies

The bioadhesion studies were based on a modification of the procedure described by Chickering and Mathiowitz (1995). PLGA/PLL-FITC samples were tested at varying concentrations of gelatin to determine the relative effects on bioadhesion for eventual reconciliation with the protein release data.The percent of gelatin ranged from 0 to 10%. Figure 3 shows a graph of the results obtained. Adhesion strength is directly proportional to the amount of gelatin present in the sample, as might be expected. These data are now of limited value; the data are of more practical import to the in vivo uptake experiments where bioadhesion will be directly related to particle uptake and eventually to immunogenicity and protection.

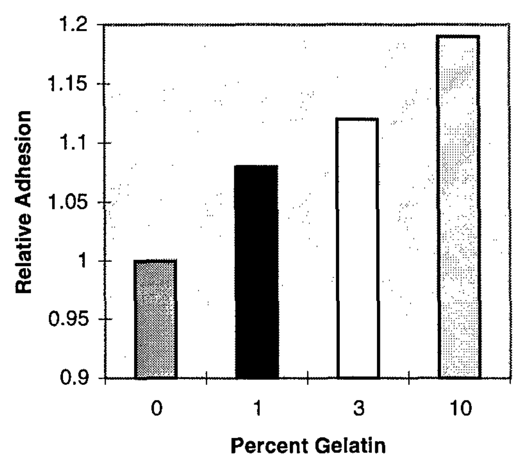


Figure 3. Graphical Representation of the Adhesion Profile with Gelatin as the Bioadhesive.

In Phase I, other bioadhesives (e.g., Eudragit) were utilized. The formulations with gelatin exhibited the strongest bioadhesion, with the poly-L-lysine protein contributing also to bioadhesion.

5.2 Particle Size Reduction

Since obtaining the correct particle size is paramount to the success of the uptake studies, a major emphasis of the first year of this Phase II SBIR has been in developing an optimized procedure for grinding the polymer to the required size distribution. Although the weight percentage of the target particles was not optimal, the initial results from the

microfluidization experiments proved very encouraging. The pressure for this set of samples varied from 22 to 5 Kpsi. The size fed into the instrument ranged from 45 to 400 microns. The polymer concentration was 0.3 g/L, and the chamber was cooled with the aid of an ice bath. It was observed that samples of 400 μm clogged the interaction chamber. Particles of less than 10 μm were obtained; however, these agglomerated around the larger particles still present. The SEMs corroborated this finding. Figures 4A and 4B showed the morphology of the polymer at different magnifications. It was also noticed that the interaction chamber got very hot while the grinding was taking place (70°C). This was of concern since degradation of the polymer and melting could occur at those temperatures with the thermoplastic PLGA polymer. An ice bath was not sufficient to cool the chamber.

Figure 4A. Scanning Electron Micrograph of PLGA after processing through a microfluidizer. Magnification = × 1000; [PLGA] = 0.3 g/L; solvent = isopropyl alcohol

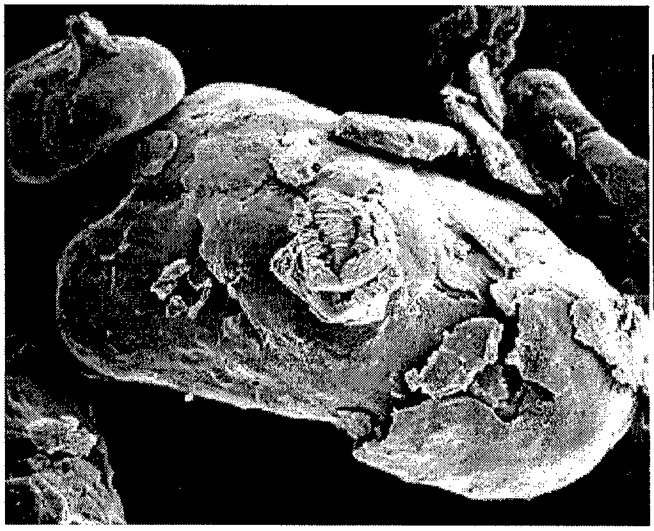


Figure 4B. Scanning electron micrograph of PLGA after processing through a microfluidizer. Magnification = × 1500; [PLGA] = 0.3 g/L; solvent = isopropyl alcohol

Several modifications were made for the next series of experiments. The solvent matrix remained the same (isopropyl alcohol). The concentration of polymer, however, was reduced to 0.1 g/L to reduce the problem of agglomeration. At a lower concentration of PLGA, surface effects are minimized an thus there is a reduced chance that a smaller particle would be drawn to a larger particle. The pressure was lowered to 5000 Psi, and the average size of the original sample was also dropped to an average size range of 45-90 μm. An ice bath was utilized again to cool the interaction chamber. The same sample was recirculated for 1 h. To determine the amount of time required to obtain polymer of adequate size, aliquots samples were observed under the microscope at six minute intervals. It was noticed that reduction of size stopped after 18 minutes, and some agglomeration was observed but less than in previous attempts. Figures 5A and 5B represent the micrographs of samples after grinding for 18 minutes (5A) and after grinding for 30 minutes (5B).

Figure 5A. SEM of PLGA after processing through a microfluidizer for 18 minutes. Magnification = × 2000; [PLGA] = 0.1 g/L; solvent = isopropyl alcohol; pressure = 5000 Psi; method of cooling = ice bath

Figure 5B. SEM of PLGA after processing through a microfluidizer for 30 minutes. Magnification = × 2000; [PLGA] = 0.1 g/L; solvent = isopropyl alcohol; pressure = 5000 Psi; method of cooling = ice bath

The temperature was a reason for concern as it increased to 70 °C during the experiment. Agglomeration of the small particles around the remaining larger particles did occur to a lesser extent. To address these problems, cryogenic cooling was used to cool the chamber. A dry ice/acetone bath (-45 °C) was utilized; the concentration of

polymer remained at 0.1 g/L. The pressure was increased to 10 kPsi; one desired outcome of increasing the pressure was an increase in the speed of grinding. Aliquot samples of solution were examined with the aid of a microscope at 6 minute intervals. Little or no agglomeration occurred as a result of the new conditions. The temperature of the PLGA/isopropyl alcohol matrix remained at -15°C throughout the run. Particle reduction was achieved in 18 minutes. Figure 6A shows the SEM of the polymer after 18 minutes of grinding through a microfluidizer, and Figure 6B shows the same after 30 minutes. No appreciable change occurred after 18 minutes.

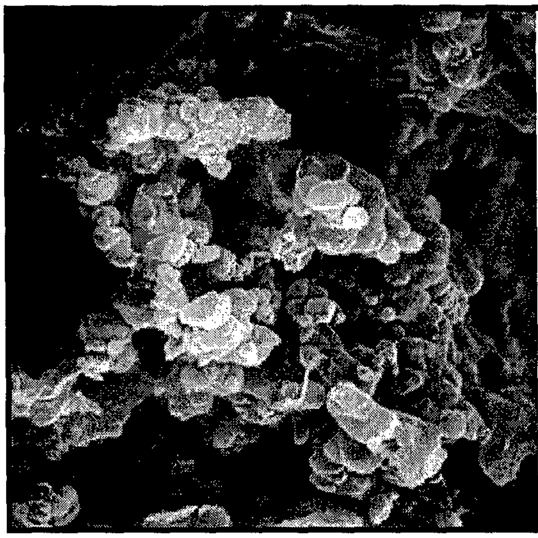


Figure 6A. SEM of PLGA after processing through a microfluidizer for 18 minutes. Magnification = × 6500; [PLGA] = 0.1 g/L; solvent = isopropyl alcohol; method of cooling= dry ice/isopropyl alcohol bath

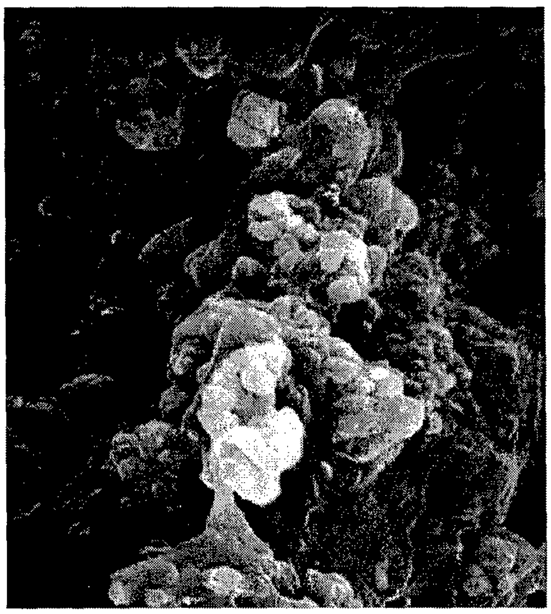


Figure 6B. SEM of PLGA after processing through a microfluidizer for 30 minutes. Magnification = × 6500; [PLGA] = 0.1 g/L; solvent = isopropyl alcohol; method of cooling= dry ice/isopropyl alcohol bath

The recovery of particles of less than 10 μm by the use of these combined particle reduction techniques is good. Table I shows the recovery of particles after utilizing the microfluidizer at 5 Kpsi.

Particle Size (microns)	Volume Fraction
45	20.90
35	16.86
25	15.36
15	19.91
7	26.97

Table I. Distribution of Particles Represented as Volume Fraction at a Temperature of 30 °C and a Pressure of 5 Kpsi.

It is apparent that particles of less than 10 μm (7 μm in this case) are in abundance. This table can be better observed as a distribution graph of volume fraction as a function of particle size. Figure 7 shows such a graph.

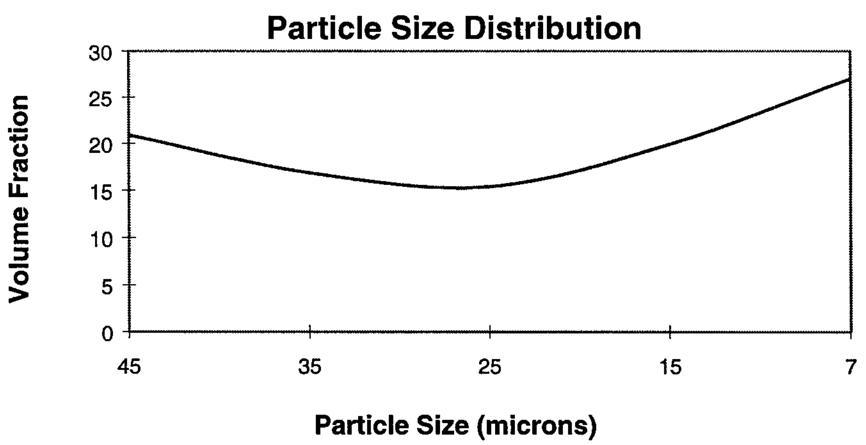


Figure 7. Graphical Representation of the Distribution of Particles After Microfluidization at 5 Kpsi

A dramatic increase in the amount of particles of less than 10 μm is observed when the temperature is decrease to -15 °C, and the pressure is increase to 10 Kpsi. Table II presents the analysis of the data obtained after grinding at such conditions.

Particle Size (microns)	Volume Fraction
45	6.53
35	15.37
25	16.81
15	12.10
7	49.19

Table II. Distribution of Particles Represented as Volume Fraction at a Temperature of -15 °C and a Pressure of 10 Kpsi.

Figure 8 shows the results graphically. Although the recovery of particles in the range of 35-15 microns remains the same, the real difference comes in the decrease in the presence of the larger particles utilizing cryogenic cooling and higher pressures.

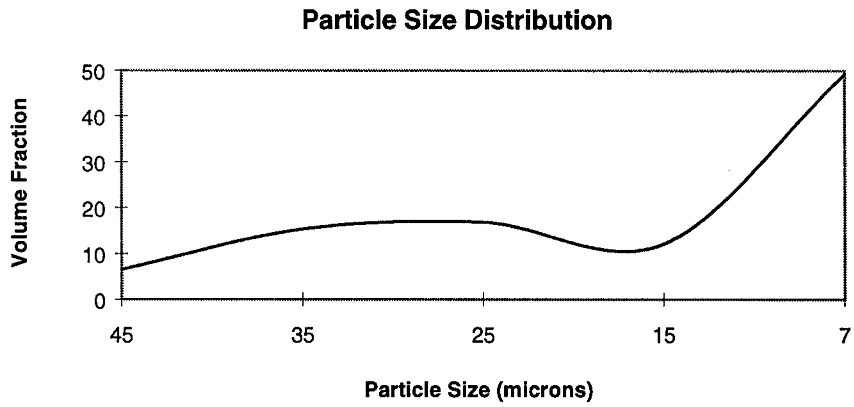


Figure 8. Graphical Representation of the Distribution of Particles After Microfluidization at 10 Kpsi

6. FUTURE WORK

The use of microfluidizer technology along with other methods of particle reduction have resolved the issue of obtaining the proper particle size for the *in vivo* uptake studies. Cambridge Scientific, Inc. is now strongly positioned to proceed with the *in vivo* bioadhesion studies. The first series of experiments will include the incorporation of the poly-L-lysine as a fluorescent label; extrusion into a rod of the matrix followed by size reduction of the rod. These studies with the fluoresecent label will be followed by the evaluation of immunogenicity of candidate antigen matrices. These formulations will include gelatin type A as the bioadhesive aid (10%). The project collaborators at OraVax, Inc. (Dr. Paul Giannasca and Dr. Thomas Monath) are assisting with this aspect of project planning.

It is unlikely that the PLGA formulation methodology proposed for the subject vaccines will prove applicable as a universal carrier for all antigens. However, it will be possible to exploit the characteristics of this PLGA-based technology to deliver vaccines for which it is best suited. The tasks proposed in this Phase II project will assist in delineating those application areas for which the subject technology will be best applied. Beginning with what we believe are two marketable applications areas based on expert scientific and marketing input, results of this Phase II project will support future product development activities at Cambridge Scientific, Inc. Further product development can be applied to both new vaccines and improved vaccines against diseases that are poorly controlled (e.g., infections caused by rotavirus, influenza virus, *Escherichia coli, Shigellae, Vibrio cholerae,* and *Salmonella typhi*). It is anticipated that successful Phase II performance will establish the feasibility of our PLGA technology for oral vaccines and allow us to reach out to additional private sector and Government markets.

Oral vaccines are more likely to be effective in protecting against mucosal infections because of their ability to induce an sIgA response. In addition to this potential immunological advantage there are practical benefits to oral vaccines. These include easier administration and 'unlimited' frequency of boosting (if necessary) owing to the fact that oral vaccines reduce the need for trained personnel to deliver the vaccines. Additionally, the avoidance of needles predicts greater product safety eliminating the possible risks of contamination and cross-infection. Moreover, an oral vaccine should ultimately be cheaper because (1) needle purchase would be eliminated, and (2) oral products traditionally require less stringent manufacturing conditions for approval. The cost of vaccines to the healthcare industry, at large, and military and developing country markets, specifically, is an important issue. The development of less expensive vaccines would have a significant impact upon the extent of vaccine coverage throughout these markets.

Vaccines are the most efficient and cost-effective means for disease prevention, but only 12% of the total costs for vaccination is to pay for the vaccine (O'Hagan, 1994). Operational costs, such as personnel training, transportation, and maintenance of the cold chain, are responsible for the remainder of the cost (Aguado and Lambert, 1992). Trained personnel would not be requires to administer oral vaccines, and this is a major advantage. Clearly, oral vaccines would be advantageous in the developing world and in the military where both would profit from increased ease of mass immunization. And, in the streamlining of the American healthcare system, cost benefits address issues of affordable immunization for all of our citizens.

We have selected two oral vaccines for Phase II studies. One would clearly have private sector commercialization potential; the other is targeted for military application. The urease system is of interest to our collaborator, OraVax, Inc. It is anticipated that successful Phase II performance would support the continued business relationship resulting in Phase III commercialization. The VEE system (or alternative, as selected by the USAMRIID) would be of interest to the Army. In this case, it is projected that success in Phase II would result in a Phase III commercialization effort with Government support. Further, then, Phase III success will allow Cambridge Scientific, Inc. to develop additional vaccine delivery products.

7. LITERATURE CITATIONS

M.T. Aguado, and P.H. Lambert, 1992, "Controlled Release Vaccines-Biodegradable Polylactide/polyglycolide (PLPG) Microspheres as Antigen Vehicles," *Immunobiology*, **184**, 113.

M.J. Alonso, S. Cohen, T.G. Park, R.K. Gupta, G.R. Siber, and R. Langer, R., 1993, "Determinants of Release Tetanus Vaccine from Polyester Microspheres," *Pharm. Res.*, **10**, 945.

H. Boeing, 1991, "Epidemiological Research in Stomach Cancer: Progress over the Last Ten Years", *J. Cancer Res. Clin. Oncol.*, **117**, 133.

L. Chen and G. N. Cyr, 1970, "Composition Producing Adhesion Through Hydration", in **Adhesive Biological Systems**, R. S. Manly, ed., Academic Press, N. Y., Chapter 10.

C.N. Chiang, L.E. Hollister, A. Kishimoto, A. and G. Barnett, 1984, "Kinetics of a Naltrexone Sustained Release Preparation", *Clin. Pharmacol. Ther.*, **36**, 704.

D.E. Chickering and E. Mathiowitz, 1995, "Bioadhesive Microspheres: I A Novel Electrobalance-baed Method to Study Adhesive Interactions Between Individual Microspheres and Intestinal Mucosa", *J. Controlled Release*, **34**, 251-261.

L. Clayton and H. Kleanhous, 1993, "Molecular Studies on the Epidemiology and Pathogenicity of ***Helicobacter Pylori***, in C. S. Goodwin and B. W. Worsley, ***Helicobacter Pylori.*** **Biology and Clinical Practice**, CRC Press, Boca Raton, FL, p. 143.

E. Cutright, B. Perez, J. D. Beasly III, W. J. Larson and W. R. Posey, 1974, "Degradation Rates of Polymers and Copolymers of Polylactic and Polyglycolic Acids," *Oral Surg.*, **37**, 142-152.

J.G. DuChamp., 1992, "Release of Isoniazid from Dry Mixed Extruded Matrices," M.S. Thesis (Chem. Eng.), Northeastern University, Boston, MA.

J. H. Eldridge, J. K. Staas, J. A. Meulbroek, et al., 1991, "Biodegradable and Biocompatible Poly (D,L-Lactide-co-Glycolide) Microspheres as an Adjuvant from Staphylococcal Enterotoxin B Toxoid which Enhances the Level of Toxin-Neutralizing Antibodies", *Infec. Imm.*, **59**, 2978-86.

J. H. Eldridge, J.K. Staas, J.A. Meulbroek, T.R.Tice, and R.M. Gilley, 1991a, "Biodegradable and Biocompatible Poly(Dl-lactide-co-glocolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid which Enhances the Level of Toxin-neutralizing Antibodies," *Infect. Immun.*, **59**, 2978.

J.H. Eldridge, J.K. Staas, J.A. Meulbroek, T.R. Tice, and R.M. Gilley, 1991b, "Biodegradable Microspheres as a Vaccine Delivery System," *Mol. Immunol.*, **28**, 287.

T.H. Ermack, E.P. Dougherty, H.R. Bhagat, Z. Kabok, and J. Pappo, 1995, "Uptake and Transport of Copolymer Biodegradable Microspheres by Rabbit Peyer's Patch M Cells," *Cell Tissue Res.*, **279**, 433.

L. Ferrero and A. Lee, 1991, "The Importance of Urease in Acid Protection for the Gastric Colonizing Bacteria ***Helicobacter Pylori*** and ***Helicobacter Felis***," Sp. Nov., *Microb. Ecol. Health Dis.*, **4**, 121.

P.R.J. Gangadharam, D.R. Ashtekar, D.C. Farhi, and D.L. Wise, 1991, "Sustained Release of Isoniazid *In vivo* from A Single Implant of a Biodegradable Polymer", *Tubercle*, **72**, 115-122.

Gelatin, 1973, product literature published by Gelatin Manufacturers Institute of America, Inc., New York.

R.M. Gilley, J.K. Staas, T.R. Tice, J.D. Morgan, and J.H. Eldridge, 1992, Encapsulation and its Application to Vaccine Development," *Proc. Int. Symp. Controlled Rel. Bioact. Mater.*, **19**, 110.

I .J. Goldstein, et al., 1980, *Nature*, **285**, 66.

S. Goodwin, J. A. Armstron, T. Chilvers, et al., 1989, "Transfer of Campylobacter Pylori and Campylobacter Mustelae to Helicobacter Gen. Nov. as ***Helicobacter Pylori*** Comb. Nov. and Helicobacter Mustelae Comb. Nov.", respectively, *Int. J Syst. Bacteriol.*, **39**, 397.

Y. Graham, 1989, "Camphylobacter Pyloridis and Peptic Ulcer Disease", *Gastroenterology*, **96**, 615.

J. D. Gresser, D. L. Wise, L.R. Beck and J.F. Howes, 1978, "Larger Animal Testing of an Injectable Sustained Release Fertility Control System", *Contraception*, **17**, 253.

J.D. Gresser, E.S.E. Hafez and W.A.A. van Cs. eds. 1980, In **Biodegradables and Delivery Systems for Contraception**, (Vol. 1, Ch. 5 of **Progress in Contraceptive Delivery Systems**, E.S.E. Hafez, series ed.) MTP Press, Lancaster, England, "Biodegradable Cylindrical Implants for Fertility Control", with D.L. Wise, L.R. Beck, and J.F. Howes.

G. Gristina, M. Oga, X. L. Webb, and C. D. Hobgood, 1983, "Adherent Bacterial Colonization in the Pathogenesis of Osteomyelitis", *Science*, **228**, 990.

L. Gross and R. Hoffman, "Medical and Biological Adhesives", 1977, in **Handbook of Adhesives**, 2nd Ed. I. Streist, ed. Van Nostrand-Reinhold, New York, p. 818.

K. Gupta, S.-H. S. Leung, and J. R. Robinson, "Bioadhesives/Mucoadhesives in Drug Delivery to the Gastrointestinal Tract", in **BioAdhesive Drug Delivery Systems**, Chapter 4, Op. Cit., p. 65.

Ibid., p. 72

R. Gurny and N. A. Peppas, 1990, "Semisolid Dosage Forms as Buccal Bioadhesives:", in **Bioadhesive Drug Delivery Systems**, Chapter 8, V. Lenearts and R. Gurny, eds., CRC Press, Boca Raton, FL.

S. Hamilton-Miller and R.A. Gargan, 1979, "Rapid Screening for Urease Inhibitors", *Invest. Urol.*, **16**, 327.

Y.-Y. Hsu, J. D. Gresser, D. J. Trantolo, C. M. Lyons, P.R.J. Gangadharam, and D. L. Wise, 1994, "*In vivo* Controlled Release of Isoniazid from Poly(lactide-co-glycolide) Matrices", *J. Contr. Release*, **31**, 223-228.

I.Ito, 1969, "Structure and Function of the Glycocalx", *Fed. Proc.*, **28**, 12,.

D .C. Kilpatrick, A. Pusztai, G. Grant, C. Garaham, and S. W. B. Ewen, 1985, "Tomato Lectin Resists Digestion in the Mammalian Alimentary Canal and Binds to Intestinal Villi Without Deleterious Effects", *FEBS Lett.*, **185**, 299-305.

E. Lefevre, J. B. Warren and D. D. Joel, 1985, "Particles and Macrophages in Murine Peyer's Patches", *Exp. Cell. Biol.*, **53**, 121-129.

C.-M. Lehr, J. A. Bouwstra, W. Kok, A. B. J. Noach, A. G. de-Boer, and H. Junginger, 1992, "Bioadhesion by Means of Specific Binding of Tomato Lectin", *Phar. Res.*, **9**, 547-553.

J. Marchelonis and G. M. Edelman, 1968, *J. Mol. Biol.*, **32**, 453.

M. Murukami, J. K. Yoo, M. Inada, et al., 1990, "Effect of Ammonia on the Gastric Mucosa in Rats: Pathophysiological Importance of Urease in Gastric Ulcer Disease", *Japan J. Pharmacol.*, **47**, 330.

R. Neutra and J. P. Kraehenbuhl, 1992, "Transepithelial Transport and Mucosal Defense; The Role of M Cells", *Trends Cell Biol.*, **2**, 134-138.

D.T. O'Hagan, H. Jeffery, H., M.J.J. Roberts, J.P. McGee, J.P., and S.S. Davis, 1991, "Controlled Release Microparticles for Vaccine Development," *Vaccine*, **9**, 768.

D.T. O'Hagan, H. Jeffery, H., and S.S.Davis, 1993, "Long Term Antibody Responses in Mice Following Subcutaneous Immunization with Ovalbumin Entrapped in Biodegradable Microparticles," *Vaccine*, **10**.

D.T. O'Hagan, 1994, "Oral Immunization and the Common Mucosal System," in **Novel Delivery Systems for Oral Vaccines**, Derek T. O'Hagan, Editor (CRC Press, Inc., Boca Raton), pp. 1-27.

L. Owen and T. H. Ermak, 1990, "Structural Specializations for Antigen Uptake and Processing in the Digestive Tract", *Semin. Immunopathol.*, **12**, 139-152.

L. Owen and D. K. Bhalla, 1983, "Cytochemical Analysis of Alkaline Phosphatase and Esterase Activities and of Lectin-Binding and Anionic Sites in Rat and Mouse Peyer's Patch M-cells", *Am. J. Anat.*, **168**, 199-212.

J. Pappo and T. H. Ermak, 1989, "Uptake and Translocation of Fluorescent Latex Particles by Rabbit Peyer's Patch Follicle Epithelium: A Quantitative Model for M Cell Uptake", *Clin. Exp. Immunol.*, **76**, 144-148

K. Park and J. R. Robinson, 1984, "Bioadhesive Polymers as Platforms for Oral-Controlled Delivery: Method to Study Bioadhesion", *Int. J. Pharm.*, **19**, 107.

K. Park, H. S. Ch'ng, and J. R. Robinson, 1985, "Alternative Approaches to Oral Controlled Delivery: Bioadhesives and *In-Situ* Systems" in **Recent Advances in Drug Delivery Systems**, J. M. Anderson and S. W. Kim, eds. Plenum Press, 163.

A. Peppas and P A. Buri, 1985, "Surface, Interfacial and Molecular Aspects of Polymer Bioadhesion on Soft Tissues", *J. Controlled Release*, **2**, 257.

W. Pritchard, 1971, "The Role of Hydrogen Boning in Adhesion", *Aspects Adhes.*, **6**, 11, 1971.

A. Pusztai, **Plant Lectins**, Cambridge University Press, Cambridge, UK , 1991.

H. Rauvala, 1983, "Cell Surface Carbohydrates and Cell Adhesion", *Trends Biochem. Sci.*, 323.

R.H. Reuning, S.H.T.Liao, A.E. Staubus, S.B. Ashcraft, D.A. Downs, S.E. Harrigan, J.N. Wiley, and D.L. Wise, 1983, "Pharmacokinetic Quantitation of Naltrexone Controlled Release from a Copolymer Delivery System", *J. of Pharmokinetics and Biopharmaceutics,* **11**, 369.

A.C. Sharon, and D.L. Wise, 1981, "Development of Drug Delivery Systems for Use in Treatment of Narcotic Addiction", in R.E. Willett and G. Barnett (Eds.), **Narcotic Antagonists Naltrexone Pharmacochemistry and Sustained Release Preparation**, NIDA Research Monograph 28, DHHS publications (SM) No. 81-902, Washington, D.C.

N. Sharon, 1991, "Molecular Basis of Lectin-Carbohydrate Interactions", in **Lectins and Cancer**, H. J. Gabius and S. Gabius, eds., Springer-Verlag, Berlin, 1.

M. Singh, A. Singh, and G. P. Talwar, 1991, "Controlled Delivery of Diphtheria Toxoid Using Biodegradable Poly(D,L-lactide) Microcapsules", *Pharm. Res.*, **8**, 958-961.

D.L. Wise, G.V. McCormick, and G.P. Willet, 1976, *Life Sciences*, **19**, 867.

D.L. Wise, J.D. Gresser, and G.J. McCormick, 1979, *J. Pharm. Pharmacol.*, **31**, 201.

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REPLY TO
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